Environmental Health Perspectives
Vol. 93, 133–144, 1991

Oncogenes and Tumor-Suppressor Genes

by Teresa A. Lehman,* Roger Reddel,* Andrea M. A. Pfeifer,* Elisa Spillare,* M. Edward Kaighn,* Ainsley Weston,* Brenda I. Gerwin,* and Curtis C. Harris*

The functional role of oncogenes in human lung carcinogenesis has been investigated by transfer of activated oncogenes into normal cells or an immortalized bronchial epithelial cell line, BEAS-2B. Transfection of v-Ha-*ras*, Ki-*ras*, or the combination of *myc* and *raf* into BEAS-2B cells produced tumorigenic cell lines, while transfection of *raf* or *myc* alone produced nontumorigenic cell lines. In addition to studying the pathogenic role of oncogenes, we are attempting to define negative growth-regulating genes that have tumor-suppressive effects for human lung carcinomas. Our strategy to identify tumor-suppressor genes involves loss of heterozygosity studies, monochromosome-cell fusion, and cell-cell fusion studies. Loss of heterozygosity studies have revealed consistent allelic DNA sequence deletions on chromosome 17p in squamous cell carcinomas, while large cell carcinomas and adenocarcinomas retained this locus. Mutations in p53, a tumor-suppressor gene located on chromosome 17p, have been observed. Cell-cell hybrid clones produced from fusion of nontumorigenic BEAS-2B cells with tumorigenic HuT292DM cells generally are nontumorigenic. The mechanistic role of the known tumor-suppressor genes *Rb*-1 and p53 in the development of human lung carcinomas is being investigated in this epithelial cell model of human bronchogenic carcinogenesis.

Introduction

Carcinogenesis has long been thought to be due to an accumulation of genetic and epigenetic changes that cause abnormal regulation of molecular control of cell growth. The genetic changes can be the activation of proto-oncogenes and/or the inactivation of tumor-suppressor genes that can initiate tumorigenesis as well as enhance its progression. For example, Ki-*ras* activation in colorectal carcinoma is considered an early event (*1*), and gene amplification of N-*myc* has been associated with progression of human neuroblastoma (*2*). To date, only two tumor-suppressor genes have been well characterized. The retinoblastoma gene, *Rb-1*, has been found to be inactivated by mutation including deletions in retinoblastomas and other human tumors (*3–5*). The other tumor-suppressor gene recently identified is p53, which is mutated in colorectal, breast, brain, and lung carcinomas and may be involved in tumor progression (*6,7*).

Oncogenes

Our strategy for investigating the role of oncogenes in the neoplastic transformation of normal human bronchial epithelial cells is shown in Table 1. Seven families of activated proto-oncogenes, *ras*, *raf*, *jun*, *erb*-B2 (*neu*), *fur*, *myb*, and *myc*, have been associated with human lung cancer. The functional role of these oncogenes in lung carcinogenesis is being studied *in vitro* by introducing these genes, singly or in combination, into normal human bronchial epithelial cells (NHBE) and SV40 T-antigen "immortalized" bronchial epithelial cells. Since the NHBE cells are the presumed progenitor cells for bronchogenic carcinoma, we have optimized their growth in culture by creating a chemically defined medium (*8*). This medium is free of serum and transforming growth factor-β_1 (TGF-β_1), which will induce terminal squamous differentiation in these cells (*9*).

Table 1. Strategy for studying neoplastic transformation of human bronchial epithelial cells by activated proto-oncogenes.

a) Select activated proto-oncogenes associated with human lung cancer
b) Transfer activated proto-oncogenes into the progenitor epithelial cells of bronchogenic carcinoma
c) Select preneoplastic and neoplastic cells from putative suppressive normal cells
d) Determine tumorigenic potential in athymic nude mice
e) Investigate dysregulation in molecular controls of growth and terminal differentiation

To study the functional involvement of Ha-*ras* in human lung carcinoma, we have transfected v-Ha-*ras* into NHBE cells (*10*). The transfected cells sustained progressive genotypic and phenotypic changes that in-

*Laboratory of Human Carcinogenesis, National Cancer Institute, Bethesda, MD 20892.

Address reprint requests to C. C. Harris, Laboratory of Human Carcinogenesis, Building 37, Room 2C01, Bethesda, MD 20892.

cluded decreased responsiveness to induction of terminal squamous differentiation, increased responsiveness to serum mitogens, increased life span, increased chromosomal aberrations and, rarely, immortality and tumorigenicity in athymic nude mice (Table 2). Therefore, neoplastic transformation of NHBE cells by Ha-*ras* is a rare event. Both the occurrence of frequent chromosomal aberrations and the lengthy cell crisis period of these transfected cells suggest that one or more unidentified events, in addition to the introduction of Ha-*ras*, may be involved in the development of the neoplastic phenotype.

Normal human cells in culture are relatively resistant to neoplastic transformation events (*11*). Several studies have indicated that immortalization is a rate-limiting step in the multistage process of *in vitro* human cell carcinogenesis (*12–14*). In order to develop an immortalized cell system for studies of carcinogenesis, we have infected NHBE cells with the SV40 large T antigen gene (*15*). Unlike the NHBE cells, these SV40 T-antigen-containing cells, e.g., the BEAS-2B cell line, became immortalized. An attractive feature of these cells for use in carcinogenesis assays is the fact that they are nontumorigenic in early passage. In addition, these cells are aneuploid and undergo squamous differentiation in response to serum or TGF-β_1 (*9*). This is illustrated in Figure 1.

Many human lung adenocarcinomas have been shown to contain activated *ras* genes, which are thought to be involved in both early and late stages of carcinogenesis (*1,16–20*). The activated *ras* gene is most frequently Ki-*ras*, but activated N-*ras* and Ha-*ras* have also been observed in lung cancer cell lines (*21*). In this laboratory, the immortalized BEAS-2B cell line has been used to define conditions under which *ras* and other oncogenes reproducibly cause neoplastic transformation.

Table 2. Progressive phenotypic and genotypic changes in normal human bronchial epithelial cells transfected with v-Ha-*ras*.

Decreased response to inducers of terminal squamous differentiation
Increased response to serum mitogens
Increased frequency of chromosomal aberrations and aneuploidy
Increased cell population doublings
Cell "crisis"
Continuous cell line
Tumorigenicity
Increased *ras* p21 expression in tumor cells
Metastasis

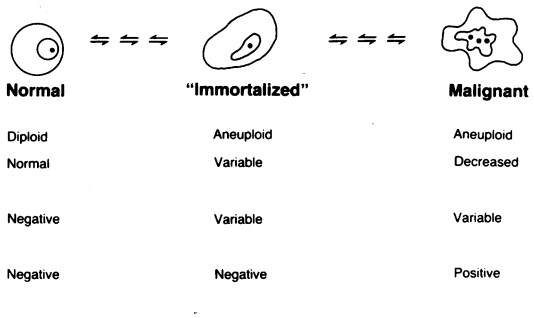

FIGURE 1. Multistep human epithelial cell carcinogenesis *in vitro*. Immortalization appears to be the rate-limiting step in *in vitro* human cell carcinogenesis.

Infection of BEAS-2B cells with a recombinant retrovirus containing v-Ha-*ras* produced cells (BZR) that were tumorigenic in athymic nude mice (*14*). Tumor analysis revealed cells of human origin with the isoenzyme phenotype and marker chromosomes of BEAS-2B cells. In addition, cell lines developed from the BZR tumors (BZRT33 and others) expressed abundant 21-kDa protein immunoreactive to antibodies specific for the codon 12 mutation present in the v-Ha-*ras* protein was autophosphorylated, indicating expression of the v-Ha-*ras* gene as opposed to an endogenous *ras* gene. BEAS-2B, BZR, and BZRT33 cells were also examined for their invasiveness, metastatic potential, and ability to repopulate de-epithelialized rat tracheal xenotransplants. Injection of these three cell lines into athymic nude mice revealed that BEAS-2B were not tumorigenic, BZR cells induced tumors with a latency period of 1 to 3 weeks, and BZRT33 induced tumors in less than 1 week (*14*). The incidence of spontaneous metastasis to the lung following subcutaneous injection was negative for BEAS-2B (0%), intermediate for BZR (33%), and extensive for BZRT33 (100%) (*14*).

Immortalized BEAS-2B cells were able to reconstitute a mucous-producing columnar epithelium in de-epithelialized rat tracheas that were transplanted subcutaneously into athymic nude mice (*22*). BZR cells were tumorigenic in this model system, and the tumor derived cell lines, e.g., BZRT33 and BZRT35 cells, that have increased ploidy and increased expression of the v-Ha-*ras* p21 protein, were more malignant than the BZR cells. This increasing malignancy in the tumor-derived cell lines correlated with increased type IV collagenase enzyme activity and mRNA expression (*22*).

The presence of an activated c-Ki-*ras* gene in human lung carcinomas has been well documented (*20,23–27*). We have investigated the role of Ki-*ras* in the multistep neoplastic transformation of human bronchial epithelial cells. The v-Ki-*ras* oncogene used for these transfections contained mutations at codons 12 and 59. The mutation at codon 12 has also been observed in the lung carcinoma cell line A549 (*28*). Transfer of this oncogene into BEAS-2B by either infection or transfection resulted in neoplastic transformation (*29*). Tumors induced by the transfection of v-Ki-*ras* had adenocarcinomatous elements (*29*). This is an interesting observation since the Ki-*ras* oncogene is most frequently found to be activated in human lung cancers, and most of these are adenocarcinomas.

Abnormalities in the *raf*, *myc*, and *ras* proto-oncogene families have been associated with both human small cell (*30–35*) and nonsmall cell lung carcinomas (*20,36–38*). We have assayed the functional role of c-*raf*-1 and c-*myc* proto-oncogenes in lung carcinogenesis by introducing these genes, both alone and in combination, into human bronchial epithelial BEAS-2B cells

(*39*). Two retroviral recombinants, p-Zip-*raf* and p-Zip-*myc*, containing the complete coding sequences of the human c-*raf*-1 and the murine c-*myc* genes, respectively, were constructed and transfected into BEAS-2B cells. BEAS-2B cells transfected with Zip-*raf* or Zip-*myc* alone were nontumorigenic after 12 months, but BEAS-2B cells transfected with Zip-*raf* and Zip-*myc* together formed large cell carcinomas in athymic nude mice in 4 to 21 weeks (*39*). Carcinomas induced by the combination of c-*raf*-1 and c-*myc* were of human epithelial origin and exhibited specific surface antigens and several neuroendocrine markers. An increase in the mRNA levels of neuron-specific enolase was detected in BEAS-2B cells containing c-*raf*-1 and c-*myc* genes, suggesting an association between transformation and the expression of several neuroendocrine markers (Pfeifer et al., unpublished results).

Tumor Suppression

The primary indication for the existence of the dominantly acting tumor-suppressor genes originates from epidemiological studies (*3*). Further evidence comes from the analysis of genetic loci exhibiting DNA restriction-fragment-length polymorphisms (RFLP) showing reduction in homozygosity of chromosome 13 in retinoblastoma and osteosarcoma (*5,40,41*) and on chromosome 11 in Wilms' tumor (*42,43*) and bladder carcinoma (*44*). These latter studies have been corroborated by genetic studies using the technique of somatic cell hybridization (*45,46*). Our strategy to identify tumor-suppressor genes involved in human lung cancer involves several approaches, which are illustrated in Table 3.

Loss of Heterozygosity

Since the location of the tumor-suppressor genes is unknown and since these genes may have different functions, a well-defined and comprehensive approach is required. An initial approach is allelic DNA sequence deletion analysis that identifies the chromosomal regions that may harbor the tumor-suppressor genes. The loss of heterozygosity (LOH) of RFLP has been used to investigate the loss of allelic DNA sequences on specific chromosomes in several types of hereditary and sporadic tumors (*40,42–44,47–51*). RFLP analysis came into prominence when the analysis of loci on 13q in hereditary retinoblastoma revealed the loss of genes on 13q. This eventually led to the identification of the *Rb*-1 gene on chromosome 13q. Recent RFLP analyses of 11p have detected loss of alleles in Wilms' tumor (*47,48*) and also in tumors associated with Beckwith-Wiedemann syndrome (*52*).

Many RFLP studies of human lung cancer have focused on small cell carcinoma (*53,54*). A small number of nonsmall cell carcinomas have been studied by DNA sequence deletion analysis (*53–55*). Recently, we have concluded an extensive analysis of nonsmall cell lung carcinoma for allelic DNA sequence losses on six different chromosomes at 13 different genetic loci. This study was conducted on tumors of varied histological types including squamous cell carcinoma, adenocarcinoma of the lung, and large cell carcinoma of the lung. This analysis allowed the comparison of the allelic DNA sequence losses in different histological classes of tumors (*56*). Interestingly, in squamous cell carcinoma, consistent LOH was found at 17p13 using the D17S1 probe, while consistent LOH at this locus in adenocarcinomas and large cell carcinomas was not detected. Frequent LOH at this locus has also been associated with colorectal (*57–59*) and small cell carcinoma of the lung (*53,55,60,61*).

LOH on chromosome 3 has been reported in small cell carcinomas (*53–55,60–62*). It has been speculated that this region contains a putative tumor-suppressor gene for small cell carcinomas of the lung (*53*). Our study of LOH for markers on chromosome 3 in approximately 60% of the tumors showed agreement with other reports that use DNA-RFLP to examine genetic loci on chromosome 3 in nonsmall cell lung carcinoma. However, LOH is substantially less than 100%, which is not in agreement with one report (*62*) in the literature.

We have studied chromosome 11 extensively for loss of alleles because it has been speculated to have at least one if not more tumor-suppressor genes. Six different loci on this chromosome have been studied. LOH was observed in 45% of the squamous cell carcinomas and adenocarcinomas studied (*56*). LOH was most frequently observed at the HBG2, insulin, and c-Ha-*ras* loci in both types of cancers. From these data, it was possible to establish two commonly deleted regions in lung cancer for this chromosome, namely, 11pter-p15.5 and 11p13-11q13 (Fig. 2). These findings are consistent with observations that describe two separate regions on chromosome 11 that may harbor tumor-suppressor genes that correspond to 11p13 in Wilms' tumor and 11pter-11p15.5 in rhabdomyosarcoma (*42,63*).

The LOH results obtained for nonsmall cell lung carcinoma show differences in the genetic deletions observed in various histological types of lung cancers; mitotic recombination was a rare cause of LOH. Inter-

Table 3. Strategy for identifying and studying tumor-suppressor genes in lung carcinogenesis.

a) Identify chromosomal location of putative tumor-suppressor genes
i. Allelic deletion analysis of tumor DNA versus germ line DNA
ii. Monochromosome cell hybrids
b) Genetic analysis of somatic cell hybrids
c) Isolate genes by subtraction library approach
i. Tumorigenic versus nontumorigenic hybrids
ii. Terminal squamous differentiation-resistant versus differentiation-sensitive cells
d) Isolate genes by insertional mutagenesis approach
e) Determine structure and function of isolated genes:
p53
Rb-1
Nm23
Others

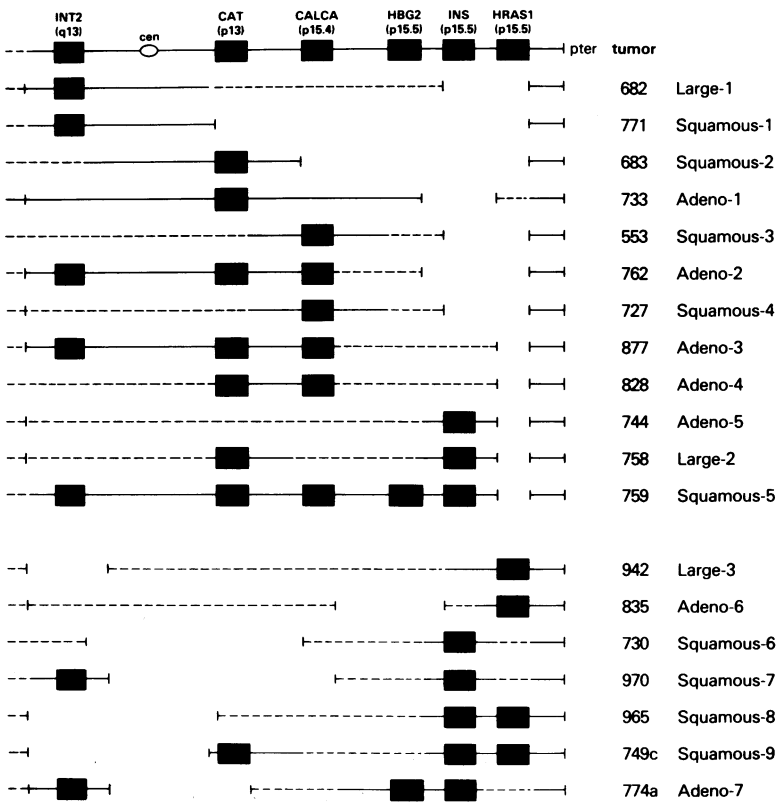

FIGURE 2. Schematic representation of shortest region of overlapping DNA sequence deletion analysis on chromosome 11 for nine squamous cell carcinomas (squamous), seven adenocarcinomas (adeno), and three large cell carcinomas (large) of the lung. Solid lines show intact genetic loci, dashed lines show regions for which no information is available, and gaps show regions of gene deletion.

estingly, in squamous cell carcinoma, coincidental LOH for several chromosomes was observed. For example, in eight of nine cases, allelic DNA sequence deletion was observed for both chromosomes 11 and 17 where the analyses were informative for both chromosomes. Similarly, allelic DNA sequence deletions occurred for chromosomes 3 and 17 in 3 of 5 informative cases. Other combinations of coincident loss in squamous and adenocarcinomas of the lung are shown in Figure 3.

The genetic changes observed in these tumors may also be involved in the pathogenesis of lung cancer in combination with other tumor-suppressor genes. From this study, we can conclude that loss of putative tumor-suppressor genes identified in other cancers may have a role independently or in combination in the development of nonsmall cell carcinoma.

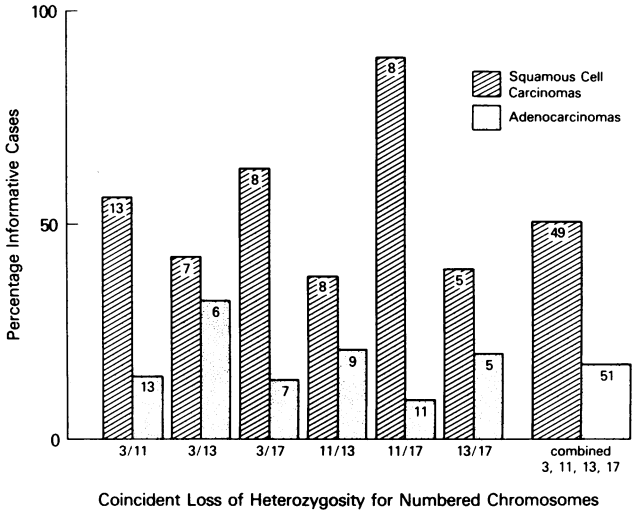

FIGURE 3. Coincident loss of heterozygosity for numbered chromosomes. For each of either 23 squamous cell or 23 adenocarcinomas of the lung, coincident loss of heterozygosity is shown for different combinations of chromosomes 3, 11, 13, and 17. Numbers in bars indicate number of cases informative for the chromosome indicated.

Monochromosome-Cell Fusion

In several cases where LOH studies suggest that deletion of a particular chromosomal region is associated with development of tumorigenicity, the technique of monochromosome fusion (*64,65*) can be employed to investigate this hypothesis. Using this technique, a single, normal human chromosome is introduced into re-

cipient tumorigenic cells. The tumorigenic potential of the microcell hybrid is assessed in athymic nude mice and in many cases, suppression of the tumorigenic phenotype is observed. For example, Stanbridge and co-workers have shown that introduction of a normal human chromosome 11 into Wilms' tumor cells suppresses tumorigenicity of these cells (*66*). Likewise, Oshimura and co-workers have observed similar suppression in cervical carcinoma cell lines (*67,68*), as has Stanbridge et al., using HeLa cells (*69*). In addition, rhabdomyosarcoma cells, which are associated with 11p15 sequence deletions, were suppressed by the monochromosome transfer of a normal human chromosome 11 (*68*). Therefore, the malignant growth of these three different types of tumor cells appear to be dependent on the absence of a gene or genes normally present on chromosome 11.

Tumor suppression has also been documented for other human chromosomes and malignant cells. Chromosome 3 has been shown to revert tumorigenicity in renal cells in which RFLP analysis has suggested that 3p deletions may be important in the development of the disease (*68*).

Most recently, Nagle and co-workers have introduced a normal human chromosome 6 into two human malignant melanoma cell lines (*70*). In addition to reversion of the transformed *in vitro* phenotype and decreased soft-agar cloning efficiency, tumorigenicity of the hybrid cells was initially suppressed. Interestingly, all animals later developed tumors. However, cytogenetic and RFLP analysis of the tumors revealed a loss of the introduced chromosome 6 from the melanoma cell hybrids (*70*).

Guided by our own LOH studies of human lung tumors and lung tumor cell lines, we are currently introducing human chromosomes 3, 11, 13, and 17 into lung cancer cell lines which are known to have sustained deletions of allelic sequences in these chromosomal regions. In addition, we are also sequentially introducing several normal chromosomes into cells that have shown multiple LOH from several different chromosomes. By taking this approach, we expect to determine if loss of one or more tumor-suppressor genes from different chromosomes may be acting in cooperation for the development of human lung carcinoma.

Cell-Cell Hybrids

Tumor suppression was first demonstrated by Harris and co-workers, who produced murine cell hybrids between cells of high and low tumorigenic potential (*71*). The tumorigenicity of these hybrids was transiently suppressed, but as the hybrid clones were propagated in culture, tumorigenic segregants rapidly developed. As chromosomes in the hybrid cells were lost, the tumorigenicity of the hybrids increased to that of the parent cell of high tumorigenic potential (*72*).

Genetic analysis of somatic cell hybrids between tumorigenic and normal human cells has shown that suppressor activity of the normal cell is functionally dominant over the tumorigenic cell. Hybrids formed from the human cervical carcinoma cell line, HeLa, and normal human fibroblasts (*73*) or normal human epidermal keratinocytes (*74*) showed suppressed tumorigenicity, as did hybrids between EJ bladder carcinoma cells containing a mutant c-Ha-*ras* and normal human fibroblasts (*75*). However, studies that examine the tumorigenicity of a cancerous cell type hybridized with its normal epithelial progenitor cell have not been performed. We therefore created cell-cell hybrids between the cancer cell line HuT292DM and NHBE, SV40 T-antigen "immortalized" nontumorigenic human bronchus cells (BEAS-2B) (*15*), or a weakly tumorigenic cell line derived from BEAS-2B which has a 3p deletion (B39TL) following growth in nude mice (Reddel et al., unpublished observations). Hybrids formed between NHBE and HuT292DM cells had a limited doubling potential in culture and senesced after 40 to 43 population doublings. Therefore, tumorigenicity assays could not be performed with these hybrids due to insufficient number of cells.

In contrast to NHBE and HuT292DM cell hybrids, hybrids of BEAS-2B and HuT292DM cells have an indefinite lifespan in culture (*76*). Tumor incidence in the parental line HuT292DM was 100% with a mean latency of 27 days, 50% in B39TL with a mean latency of 148 days, and 0% in BEAS-2B after approximately 1 year. Hybrids of BEAS-2B and HuT292DM cells yielded total suppression of tumorigenicity in 76% of the mice injected, while the immortalized, weakly tumorigenic B39TL as a parent yielded only 54% suppression of tumorigenicity of HuT292DM. Tumorigenicity of the B39TL × HuT292DM cell hybrids is comparable to the tumorigenicity of the parent B39TL at 50% (7/14). In addition, latency of tumor development in BEAS-2B × HuT292DM cell hybrids was extended 2- to 3-fold over that of the parent HuT292DM. These data are presented in Table 4.

Cell lines were isolated from tumors arising from the BEAS-2B × HuT292DM cell hybrids and the B39TL × HuT292DM hybrids. Upon reinjection of these lines into athymic nude mice, tumors were produced with latency periods comparable to the parent HuT292DM cells. These data suggest that reversion to tumor-forming ability may occur due to loss of a chromosome(s) that harbors a tumor-suppressor gene(s).

Karyotype analysis of parental lines, cell-cell hybrids, and hybrid-derived tumor cell lines was performed, and the results are shown in Table 5. The parental lines are hypodiploid, while the hybrid lines are hypotriploid to hypotetraploid. The hybrid lines contained all the marker chromosomes of both parents. In addition, new marker chromosomes were present in the hybrid tumor-derived cell lines as well as a loss of the Y chromosome from B39TL in the B39TL × HuT292DM hybrid tumor cell line. Karyotype analysis of the hybrid tumor cell lines revealed varied chromosome counts, mostly in the triploid range, suggesting a loss of chromosomes from

Table 4. Suppression of tumorigenicity in somatic cell hybrids between a lung cancer cell line and immortalized bronchial epithelial cells.

Cell line	No. of injected mice	Tumors/no. of injected mice[a]	Latency, (days) %[b]	% Totally suppressed	No. regressed[c]
BEAS-2B	15	0/12	> 294	100	0
B39TL	15	7/14	148	50	3
HuT292DM	20	19/19	27	0	0
BEAS-2B × HuT292DM[d]	55	13/54	88[e]	76	1
B39TL × HuT292DM[f]	30	13/28	83[g]	54	3

[a]A nonregressing nodule ≥ 1.0 cm in the largest dimension. Mice surviving less than 3 months without tumors have been excluded.
[b]Mean number of days to reach scorable size.
[c]Not scored as tumors.
[d]Pooled data from 11 hybrid lines each injected into mice.
[e]Mean tumor latency in the remaining 24% of the hybrids that produced tumors.
[f]Pooled data from six hybrid lines each injected into five mice.
[g]Mean tumor latency in the remaining 46% of the hybrids that produced tumors.

Table 5. Chromosomal characteristics of hybrids and parental lines.

Cell line	Ploidy[a]	Marker chromosomes: HuT292 DM	BEAS-2B	B39 TL	New	Y-chromosome
Hybrids						
HuT292DM × BEAS-2B-1, P14	75-85 (92)[b]	13	9		6	Present
HuT292DM × BEAS-2B-2, P10	75-90 (95)	13	8			Present
HuT292DM × B39TL-1, P12	80-90 (94)	12		8	3	Present
HuT292DM × B39TL-2, P10	75-90 (98)	15		7	3	Present
Tumor lines						
HuT292DM × B39TL-T, P4	65-85 (93)	7		9	10	Absent
HuT292DM × BEAS-2B-T, P4	68-78 (92)	7	2		5	Present
Parental lines						
HuT292DM	43-45 (96)	7				Absent
BEAS-2B, P27	44-48 (85)		6			Present
B39TL, P3	40-47 (90)		4	7		Present

[a]Range of chromosome numbers (% in range) based on counts of 100 metaphases/cell line.
[b]The remaining metaphases have 120 to 150 chromosomes.

the hypotriploid to hypotetraploid range observed in the hybrids.

From these experiments, we can conclude that nontumorigenic or weakly tumorigenic parents in a cell-cell hybrid with tumorigenic cells will dominantly control culture longevity and tumorigenicity of the more tumorigenic parent. Further, genes other than those involved in senescence can exhibit tumor suppressor activity.

Retinoblastoma

Retinoblastoma is a childhood cancer that occurs in familial and spontaneous forms. In 1971, Knudson proposed that this retinal cancer is caused by two mutational events (*41*). In the familial form, a germ-line mutation predisposes the individual to retinoblastoma, and a second mutation is acquired somatically, leading to tumor development (*41*). In the spontaneous form of retinoblastoma, both mutations are somatic in origin. Further, those with the hereditary form are at risk for developing secondary cancers later in life. These second cancers are of unusual types such as osteosarcoma and fibrosarcoma. Individuals with the nonhereditary form are at no increased risk for other cancers. The evidence that one of these mutations creates an inactive allele was provided by the loss of genetic material on chromosome 13q14 in retinoblastomas (*77*). This also suggested that this region harbors a gene, *Rb*-1, that serves as the first target for inactivation by these mutations. The second of Knudson's hypothesized target genes was soon identified to be the other copy of the intact *Rb*-1 gene. This was recognized by studying a closely linked marker gene, esterase D, on chromosome 13. LOH studies revealed that the esterase D gene was heterozygous in normal tissue of a retinoblastoma patient, but in the tumor cells, it was reduced to a homozygous state. This implied that in tumor cells, the intact *Rb*-1 gene was replaced by a copy of the mutated allele. This demonstrated that both copies of the *Rb*-1 gene need to be lost or inactivated for tumor development. Using RFLP techniques and chromosome walking, a candidate gene for Rb-1 has been isolated and cloned (*5,78,78*). It has been further shown that the *Rb*-1 protein is present in normal retinoblasts but absent in retinoblastomas.

All of the evidence collected to date suggests that the Rb-1 protein acts as a negative regulator of cell proliferation (*80–82*). If this is true, the Rb-1 protein must

be posttranslationally regulated. It has recently been shown that the phosphorylation level of Rb-1 changes rapidly, suggesting that specific kinases and phosphatases are involved. In addition, phosphorylation of Rb-1 is linked to the cell cycle (*83–86*). Although synthesis of the Rb-1 protein is relatively constant throughout the cell cycle, phosphorylated Rb-1 protein can be detected in cells in late G_1 and S phase, while cells in G_0 and early G_1 are less phosphorylated. The state of phosphorylation of the Rb-1 protein may act as a "gate" to allow cells to enter S phase and proliferate. In contrast, unphosphorylated Rb-1 protein may inhibit cell proliferation and enhance differentiation.

Further evidence that the unphosphorylated form of Rb-1 protein inhibits cell proliferation comes from work by Ludlow (*87*) who demonstrated that SV40 T antigen binds only to the unphosphorylated form of the Rb-1 protein. This binding may functionally inactivate the unphosphorylated form of Rb-1 by removing its regulatory effects on the cell cycle and promoting cell proliferation. The functional inactivation of Rb-1 by SV40 T-binding may correspond to the "second hit" of Knudson's hypothesis, thereby increasing the neoplastic potential of these infected cells. This regulation may be a key step in modulation of cell growth mediated by the Rb-1 protein.

Several studies have shown that nuclear viral oncogene products from adenovirus E1A (*88,89*), SV40 T-antigen (*87,90*) (as discussed above), and HPV16 E7 (*91,92*) bind to the Rb-1 protein. The importance of these interactions has not been conclusively demonstrated thus far. However, mutations in the Rb-1 binding regions of the viral protein E1A (*89*) and SV40 T-antigen (*90*) prevent the association of viral oncogene and *Rb*-1 gene products. This has been hypothesized to prevent entry of the virus-infected cells into S-phase of the cell cycle, thus preventing viral DNA replication. When viral DNA replication is prevented, the oncogenic effects of the virus are not expressed, and *Rb*-1 acts as a suppressor of cellular transformation.

Several different abnormalities have been observed in the *Rb*-1 gene and its product in retinoblastoma, osteosarcoma, small cell lung carcinomas (SCLC), breast and bladder carcinomas. These abnormalities include point mutations altering splicing patterns of mRNA, small deletions or duplications, truncations of the protein, and abnormal levels of the *Rb*-1 transcript. In 50% of human retinoblastoma tumors, point mutations which either alter the splicing pattern or generate small deletions or duplications in the gene were observed (*93,94*).

Inactivation of the *Rb*-1 gene may be involved in the development of lung cancers as well, especially in the case of SCLC. In 60% of SCLC studied, no detectable *Rb*-1 transcript was observed, while 10% of the non-SCLC had abnormal or absent *Rb*-1 transcripts (*34,95*). All SCLC examined for Rb-1 protein were found to be negative (*95*). One of four pulmonary carcinoids examined had *Rb*-1 structural abnormalities, while three expressed no *Rb*-1 mRNA (*34*).

Our approach has been to examine various lung cancer and mesothelial cell lines for *Rb*-1 abnormalities by Northern blot analysis and by immunoprecipitation. This will allow identification of cell lines with defective *Rb*-1 genes for further characterization of *Rb*-1 involvement in lung cancer and mesotheliomas. Once these lines have been identified, the *Rb*-1 gene can be introduced by DNA transfection or by microcell fusion with a cell line containing a marked normal human chromosome 13 to determine biological effects of the *Rb*-1 gene in these cells.

One recent demonstration of tumor suppression by *Rb*-1 was shown by introducing the *Rb*-1 gene into a tumorigenic cell line which lacks the gene and then examining changes in growth and tumorigenic potential. Lee and co-workers (*82*) have shown that introduction of the cDNA from *Rb*-1 into a retinoblastoma cell line that lacks the Rb-1 protein as well as an osteogenic sarcoma line expressing a truncated Rb-1 protein greatly inhibited growth in culture and the ability to grow in an anchorage-independent manner. Furthermore, the tumorigenic potential of the retinoblastoma and osteogenic sarcoma cell lines was lost in the cells which now contained the *Rb*-1 gene (*82*). However, introduction of the same *Rb*-1 gene construct into the human prostate cell line DU145, which has a 35 amino acid in-frame deletion, did not significantly alter its genetic growth rate in culture (*96*). Unlike the retinoblastoma and osteosarcoma cell lines containing *Rb*-1, the tumorigenicity was not lost, but the tumor sizes were greatly reduced in the mice injected with the prostate cell line containing the *Rb*-1 gene (*96*).

p53

Phosphoprotein p53 is a nuclear protein that is present in high amounts in transformed human (*97*) and mouse cells (*98*). Although no specific function has been assigned to this protein, antibody injections into dividing cells have implicated p53 in cell cycle regulation (*99*). Initial studies in rat embryo fibroblasts have shown that p53 can cooperate with *ras* in neoplastic transformation (*100*). Recently, it has been shown that the p53 gene used in this and other studies was mutated, and not the wild type gene (*101*). In fact, it has been recently demonstrated that wild-type p53 does not cooperate with *ras*, but suppresses focus formation when co-transfected with *ras* in this assay (*102,103*).

One of the best-characterized features of p53 is its ability to form complexes with other proteins. p53 was first identified in a complex with SV40 T-antigen (*98,104*). Since that time, it has been found associated with adenovirus E1b in transformed rodent cells (*105*) and HPV16 E6 (P. Howley, personal communication). In addition, p53 complexes with itself to form homo-oligomeric structures (*106*).

In situ hybridization analysis has assigned the p53 gene to the short arm of human chromosome 17, banding region 13 (*107*). As discussed above, several recent RFLP studies in human lung carcinoma, breast carcinoma, colorectal carcinoma, and brain tumors have

shown LOH in this region of the chromosome. This finding led to the hypothesis that this region harbors a tumor-suppressor gene. In a recent study, Vogelstein and co-workers have shown that in two colorectal carcinomas, one of the 17p alleles is lost and the p53 gene on the other is mutated, while normal tissue surrounding the tumor has the p53 wild-type sequence (*6*). This finding has sparked speculation that progression of these tumors occurs through a dominant, negative effect mediated by the presence of mutant p53 or complete loss of wild-type p53 (*6,102,108*). A dominant negative effect may occur when pseudohomodimers of wild-type and mutated p53 are formed which functionally inactivate the wild type p53 (*109*).

To further explore the possible dominant negative effect of mutant p53, Bernstein et al. (*110*) have generated independent lines of transgenic mice carrying genomic clones of a mutant p53 gene. These mice expressed high levels of mutant p53 in a wide variety of tissues and have a greatly elevated predisposition to malignancies, particularly osteosarcomas, lung adenocarcinomas, and lymphomas (*110*). Both alleles of the p53 gene used to develop the transgenic mice have sustained mutation in the coding region. The elevated tumor incidence in mice could be due to a dominant negative effect of functionally inactive transgenic protein inhibiting normal endogenous wild-type p53 protein.

Previous studies in rodent systems have shown that mutant p53 binds to cellular heat shock protein 70 (hsp70) (*109*). Immunoprecipitation using hsp antisera or p53 antibodies has clearly demonstrated this complex formation, which results from conformational changes in the p53 due to mutation. The association of p53 with hsp70 in a human system has recently been demonstrated (*111*). Cell lysates of a human osteosarcoma cell line, HOS-SL, were immunoprecipitated with anti-hsp70 and anti-p53 antibodies, and co-immunoprecipitation of p53 and hsp70 was observed. Subsequent cloning and sequencing of the p53 gene has revealed a mutation in codon 156 of the p53 gene (*112*). We are currently using co-immunoprecipitation with hsp70 as a rapid method of screening cell lines for mutations in the p53 gene.

In the last year, there have been several reports regarding p53 mutations detected in human tumors and cell lines (*6,7,113*). A wide range of abnormalities of the p53 gene, its RNA, and protein products have been reported in human lung cancer cell lines. A panel of human SCLC and non-SCLC cell lines have been examined as well as samples from normal lung obtained at the time of surgical resection. Of the 30 lung cell lines examined, one had a DNA rearrangement, 4 had abnormally sized p53 mRNA, 4 had decreased levels of p53 mRNA, 2 had only trace amounts of p53 mRNA, and 10 had point mutations (*113*).

Using the LOH studies on chromosome 17 as background, 21 tumors of various histological types (colorectal, lung, breast, and brain) have been analyzed for mutations in the p53 gene (*7*). Fifteen of the tumors contained a single missense mutation, two contained two missense mutations, one tumor had a frame-shift mutation, and in three tumors, no p53 mutations were detected. The mutations identified in this study were clustered in four regions, hot spots of the p53 gene. These regions, exons 5, 6, and 7, are the most highly conserved among species (*114*). Although more data are needed, these initial results suggest that these regions of the p53 gene may be especially important in mediation of the normal function of the p53 gene product. Normal cells from tissue surrounding these tumors were also analyzed for p53 mutations, and none were found.

Wild-type p53 as a putative tumor-suppressor gene has many properties in common with *Rb*-1, the only other known tumor-suppressor gene. A comparison of the characteristics and activities of the *Rb*-1 and p53 gene products is shown in Table 6. Both these genes encode nuclear phosphoproteins that bind DNA and have a possible regulatory function in the cell cycle. Most notably, both of these proteins form complexes with oncoproteins of DNA tumor viruses. The binding regions of these oncoproteins to *Rb*-1 and p53 are shown in Figure 4. As was discussed earlier in the case of *Rb*-1, these nuclear oncoproteins participate in transformation through at least one common mechanism, namely, binding to and thereby inactivating *Rb*-1 and/or p53. Since p53 is believed to be involved in transition of cells from G_1 to the S-phase of the cell cycle, by binding to the p53 protein, SV40 T-antigen would inactivate this function of p53 in the cell cycle, promote the replication of viral DNA and cause transformation.

We are examining the status of p53 in primary lung tumors compared to surrounding normal tissue and in lung carcinoma cell lines. Several different approaches are being taken. In the first approach, we are sequencing exons 5, 6, and 7 using intron primers to amplify the DNA. The polymerase chain reaction product is sequenced and examined for mutations.

The second approach takes advantage of the association of mutated p53 and heat shock proteins (*115*). Using antibodies against both p53 and heat shock proteins, immunoprecipitations of the various cell lines are performed, and the presence of mutated p53 is detected by the co-immunoprecipitation of the p53-hsp complex. As lung cancer cell lines which contain a mutated p53 are identified, they are then transfected with a variety of plasmids containing wild-type p53 either constitutively or inducibly expressed. In addition, NHBE and T-antigen-immortalized BEAS-2B cells are also transfected

Table 6. Comparison of characteristics, activities, and functions of Rb-1 and p53 proteins.

Rb-1	p53
DNA binding activity	DNA binding activity
Nuclear phosphoprotein	Nuclear phosphoprotein
Binds SV40 T-antigen	Binds SV40 T-antigen
Binds adenovirus Ela	Binds adenovirus Elb
Binds HPV-16 E7	Binds HPV-16 E6
Regulates transcription of cellular genes involved in growth control	Regulates G_1-S transition in normal cells

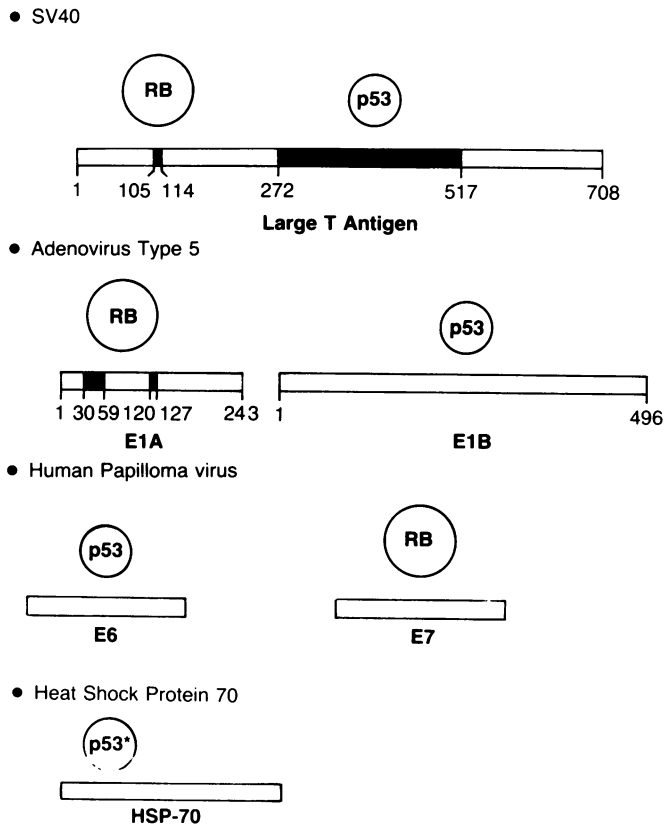

FIGURE 4. Schematic representation of the interaction between the Rb-1 protein, p53, viral proteins, and heat shock protein 70.

with wild-type and mutated p53 in constitutive or inducible expression vectors. The tumorigenicity of these transfected cells, as well as growth characteristics, will be determined. These experiments are designed to provide data that may give us some insight into the biological effects of mutated and wild-type p53 in lung cells.

REFERENCES

1. Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., and Bos, J. L. Genetic alterations during colorectal-tumor development. N. Engl. J. Med. 319: 525–532 (1988).
2. Brodeur, G. M., Hayes, F. A., Green, A. A., Casper, J. T., Wasson, J., Wallach, S., and Seeger, R. C. Consistent N-*myc* copy number in simultaneous or consecutive neuroblastoma samples from sixty individual patients. Cancer Res. 47: 4248–4253 (1987).
3. Knudson, A. G., Jr. Hereditary cancer, oncogenes, and antioncogenes. Cancer Res. 45: 1437–1443 (1985).
4. Hansen, M. F., Koufos, A., Gallie, B. L., Phillips, R. A., Fodstad, O., Brogger, A., Gedde-Dahl, T., and Cavenee, W. L. Osteosarcoma and retinoblastoma: a shared chromosomal mechanism revealing recessive predisposition. Proc. Natl. Acad. Sci. U.S.A. 82: 6216–6220 (1985).
5. Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., Albert, D. M., and Dryja, T. P. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 323: 643–646 (1986).
6. Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., vanTuinen, P., Ledbetter, D. H., Barker, D. F., and Nakamura, Y. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 244: 217–221 (1989).
7. Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F. S., Weston, A., Modali, R., Harris, C. C., and Vogelstein, B. p53 gene mutations occur in diverse human tumor types. Nature 342: 705–708 (1989).
8. Lechner, J. F., and LaVeck, M. A. A serum-free method for culturing normal human bronchial epithelial cells at clonal density. J. Tissue Culture Methods 9: 43–48 (1985).
9. Ke, Y., Reddel, R. R., Gerwin, B. I., Miyashita, M., McMenamin, M. G., Lechner, J. F., and Harris, C. C. Human bronchial epithelial cells with integrated SV40 virus T antigen genes retain the ability to undergo squamous differentiation. Differentiation 38: 60–66 (1988).
10. Yoakum, G. H., Lechner, J. F., Gabrielson, E. W., Korba, B. E., Malan-Shibley, L., Willey, J. C., Valerio, M. G., Shamsuddin, A. K. M., Trump, B. F., and Harris, C. C. Transformation of human bronchial epithelial cells transfected by Harvey *ras* oncogene. Science 227: 174–1179 (1985).
11. DiPaolo, J. A. Relative difficulties in transforming human and animal cells in vitro. J. Natl. Cancer Inst. 70: 3–8 (1983).
12. Rhim, J. S., Trimmer, R., Arnstein, P., and Huebner, R. J. Neoplastic transformation of chimpanzee cells induced by adenovirus type 12—simian virus 40 hybrid virus. Proc. Natl. Acad. Sci. U.S.A. 78: 313–317 (1981).
13. Namba, M., Nishitani, K., Fukushima, F., Kimoto, T., and Nose, K. Multistep process of neoplastic transformation of normal human fibroblasts by 60Co gamma rays and Harvey sarcoma viruses. Int. J. Cancer 37: 419–423 (1986).
14. Amstad, P., Reddel, R. R., Pfeifer, A., Malan-Shibley, L., Mark, G. E., and Harris, C. C. Neoplastic transformation of a human bronchial epithelial cell line by a recombinant retrovirus encoding viral harvey *ras*. Mol. Carcinog. 1: 151–160 (1988).
15. Reddel, R. R., Ke, Y., Gerwin, B. I., McMenamin, M. G., Lechner, J. F., Su, R. T., Brash, D. E., Park, J. B., Rhim, J. S., and Harris, C. C. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res. 48: 1904–1909 (1988).
16. Guerrero, I., Calzada, P., Mayer, A., and Pellicer, A. A molecular approach to leukemogenesis: mouse lymphomas contain an activated c-ras oncogene. Proc. Natl. Acad. Sci. U.S.A. 81: 202–205 (1984).
17. Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zanca, D., and Barbacid, M. Direct mutagenesis of Ha-ras-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature 315: 382–385 (1985).
18. Bondy, G. P., Wilson, S., and Chambers, A. F. Experimental metastatic ability of H-ras-transformed NIH3T3 cells. Cancer Res. 45: 6005–6009 (1985).
19. Kasid, A., Lippman, M. E., Papageorge, A. G., Lowy, D. R., and Gelmann, E. P. Transfection of v-*ras*H DNA into MCF-7 human breast cancer cells bypasses dependence on estrogen for tumorigenicity. Science 228: 725–728 (1985).
20. Rodenhuis, S., Van de Wetering, M. L., Mooi, W. J., Evers, S. G., van Zandwijk, N., and Bos, J. L. Mutational activation of the K-ras oncogene. A possible pathogenetic factor in adenocarcinoma of the lung. N. Engl. J. Med. 317: 929–935 (1987).
21. Yuasa, Y., Gol, R. A., Chang, A., Chiu, I. M., Reddy, E. P., Tronick, S. R., and Aaronson, S. A. Mechanism of activation of an N-ras oncogene of SW-1271 human lung carcinoma cells. Proc. Natl. Acad. Sci. U.S.A. 81: 3670–3674 (1984).
22. Bonfil, R. D., Reddel, R. R., Ura, H., Reich, R., Fridman, R., Harris, C. C., and Klein-Szanto, A. J. Invasive and metastatic potential of a v-Ha-*ras*-transformed human bronchial epithelial cell line. J. Natl. Cancer Inst. 81: 587–594 (1989).
23. Der, C. J., Krontiris, T. G., and Cooper, G. M. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the ras genes of Harvey and Kirsten sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. 79: 3637–3640 (1982).
24. Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Aaronson,

S. A., and Barbacid, M. Oncogenes in solid human tumours. Nature 300: 539–542 (1982).
25. Capon, D. J., Seeburg, P. H., McGrath, J. P., Hayflick, J. S., Edman, U., Levinson, A. D., and Goeddel, D. V. Activation of Ki-ras2 gene in human colon and lung carcinomas by two different point mutations. Nature 304: 507–513 (1983).
26. Shimizu, K., Birnbaum, D., Ruley, M. A., Fasano, O., Suard, Y., Edlund, L., Taparowsky, E., Goldfarb, M., and Wigler, M. Structure of the Ki-ras gene of the human lung carcinoma cell line Calu-1. Nature 304: 497–500 (1983).
27. Santos, E., Martin-Zanca, D., Reddy, E. P., Pierotti, M. A., Della Porta, G., and Barbacid, M. Malignant activation of a K-ras oncogene in lung carcinoma but not in normal tissue of the same patient. Science 223: 661–664 (1984).
28. Valenzuela, D. M., and Groffen, J. Four human carcinoma cell lines with novel mutations in position 12 of c-K-*ras* oncogene. Nucleic Acids Res. 14: 843–852 (1986).
29. Reddel, R. R., Ke, Y., Kaighn, M. E., Malan-Shibley, L., Lechner, J. F., Rhim, J. S., and Harris, C. C. Human bronchial epithelial cells neoplastically transformed by v-Ki-*ras*: altered response to inducers of terminal squamous differentiation. Oncogene Res. 3: 401–408 (1988).
30. Little, C. D., Nau, M. M., Carney, D. N., Gazdar, A. F., and Minna, J. D. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. Nature 306: 194–196 (1983).
31. Nau, M. M., Brooks, B. J., Jr., Battey, J. F., Sausville, E., Gazdar, A. F., Kirsch, I. R., McBride, O. W., Bertness, V., Hollis, G. F., and Minna, J. D. L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature 318: 69–73 (1985).
32. Graziano, S. L., Cowan, B. Y., Carney, D. N., Bryke, C. R., Mitter, N. S., Johnson, B. E., Mark, G. E., Planas, A. T., Catino, J. J., Comis, R. L., and Poiesz, B. J. Small cell lung cancer cell line derived from a primary tumor with a characteristic deletion of 3p. Cancer Res. 47: 2148–2155 (1987).
33. Nakano, H., Yamamoto, F., Neville, C., Evans, D. A., Mizuno, T., and Perucho, M. Isolation of transforming sequences of two human lung carcinomas: structural and functional analysis of the activated c-K-ras oncogenes. Proc. Natl. Acad. Sci. U.S.A. 81: 71–75 (1984).
34. Harbour, J. W., Lai, S. L., Whang-Peng, J., Gazdar, A. F., Minna, J. D., and Kaye, F. J. Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. Science 241: 353–357 (1988).
35. Graziano, S. L., Mark, G. E., Murray, C., Mann, D. L., Ehrlich, G. D., Poiesz, B. J., and Weston, A. DNA restriction fragment length polymorphisms at either end of the c-*raf*-1 locus at 3p25. Oncogene Res. 3: 99–103 (1988).
36. Cline, M.J., and Battifora, H. Abnormalities of protooncogenes in non-small cell lung cancer correlations with tumor type and clinical characteristics. Cancer 60: 2669–2674 (1987).
37. Kurzrock, R., Gallick, G. E., and Gutterman, J. U. Differential expression of p21ras gene products among histological subtypes of fresh primary human lung tumors. Cancer Res. 46: 1530–1534 (1986).
38. Rodenhuis, S., Slebos, R. J., Boot, A. J., Evers, S. G., Mooi, W. J., Wagenaar, S. S., van Bodegom, P. C., and Bos, J. L. Incidence and possible clinical significance of K-ras oncogene activation in adenocarcinoma of the human lung. Cancer Res. 48: 5738–5741 (1988).
39. Pfeifer, A., Mark, G. E., Malan-Shibley, L., Graziano, S. L., Amstad, P., and Harris, C. C. Cooperation of c-*raf*-1 and c-*myc* protooncogenes in the neoplastic transformation of SV40 T-antigen immortalized human bronchial epithelial cells. Proc. Natl. Acad. Sci. U.S.A. 86: 10075–10079 (1989).
40. Cavenee, W. K., Dryja, T. P., Phillips, R. A., Benedict, W. F., Godbout, R., Gallie, B. L., Murphree, A. L., Strong, L. C., and White, R. L. Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature 305: 779–784 (1983).
41. Knudson, A. G., Jr. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. U.S.A. 68: 820–823 (1971).
42. Koufos, A., Hansen, M. F., Lampkin, B. C., Workman, M. L., Copeland, N. G., Jenkins, N. A., and Cavenee, W. K. Loss of alleles at loci on human chromosome 11 during genesis of Wilms' tumour. Nature 309: 170–172 (1984).
43. Orkin, S. H., Goldman, D. S., and Sallan, S. E. Development of homozygosity for chromosome 11p markers in Wilms' tumour. Nature 309: 172–174 (1984).
44. Fearon, E. R., Feinberg, A. P., Hamilton, S. H., and Vogelstein, B. Loss of genes on the short arm of chromosome 11 in bladder cancer. Nature 318: 337–380 (1985).
45. Stanbridge, E. J., Der, C. J., Doersen, C. J., Nishimi, R. Y., Peehl, D. M., Weissman, B. E., and Wilkinson, J. E. Human cell hybrids: analysis of transformation and tumorigenicity. Science 215: 252–259 (1982).
46. Sager, R. Genetic suppression of tumor formation. Adv. Cancer Res. 44: 43–68 (1985).
47. Fearon, E. R., Vogelstein, B., and Feinberg, A. P. Somatic deletion and duplication of genes on chromosome 11 in Wilms' tumours. Nature 309: 176–178 (1984).
48. Koufos, A., Hansen, M. F., Copeland, N. G., Jenkins, N. A., Lampkin, B. C., and Cavenee, W. K. Loss of heterozygosity in three embryonal tumours suggests a common pathogenetic mechanism. Nature 316: 330–334 (1985).
49. Reeve, A. E., Housiaux, P.J., Gardner, R. J., Chewings, W. E., Grindley, R. M., and Millow, L. J. Loss of a Harvey ras allele in sporadic Wilms' tumour. Nature 309: 174–176 (1984).
50. Zbar, B., Brauch, H., Talmadge, C., and Linehan, M. Loss of alleles of loci on the short arm of chromosome 3 in renal cell carcinoma. Nature 327: 721–724 (1987).
51. Lundberg, C., Skoog, L., Cavenee, W. K., and Nordenskjold, M. Loss of heterozygosity in human ductal breast tumors indicates a recessive mutation on chromosome 13. Proc. Natl. Acad. Sci. U.S.A. 84: 2372–2376 (1987).
52. Sotelo-Avila, C., Gonzalez-Crussi, F., and Fowler, J. W. Complete and incomplete forms of Beckwith-Wiedemann syndrome: their oncogenic potential. J. Pediatr. 96: 47–50 (1980).
53. Naylor, S. L., Johnson, B. E., Minna, J. D., and Sakaguchi, A. Y. Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. Nature 329: 451–454 (1987).
54. Yokota, J., Wada, M., Shimosato, Y., Terada, M., and Sugimura, T. Loss of heterozygosity on chromosomes 3, 13, and 17 in small-cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. Proc. Natl. Acad. Sci. U.S.A. 84: 9252–9256 (1987).
55. Brauch, H., Johnson, B., Hovis, J., Yano, T., Gazdar, A. F., Pettengill, O. S., Graziano, S. L., Sorenson, G. D., Poiesz, B. J., Minna, J., Linehan, M., and Zbar, B. Molecular analysis of the short arm of chromosome 3 in small-cell and non-small-cell carcinoma of the lung. N. Engl. J. Med. 317: 1109–1113 (1987).
56. Weston, A., Willey, J. C., Modali, R., Sugimura, H., McDowell, E. M., Resau, J., Light, B., Haugen, A., Mann, D. L., Trump, B. F., and Harris, C. C. Differential DNA sequence deletions from chromosomes 3, 11, 13 and 17 in squamous cell carcinoma, large cell carcinoma and adenocarcinoma of the human lung. Proc. Natl. Acad. Sci. U.S.A. 86: 5099–5103 (1989).
57. Fearon, E. R., Hamilton, S. R., and Vogelstein, B. Clonal analysis of human colorectal tumors. Science 238: 193–197 (1987).
58. Okamoto, M., Sasaki, M., Sugio, K., Sato, C., Iwama, T., Ikeuchi, T., Tonomura, A,. Sasazuki, T., and Miyaki, M. Loss of constitutional heterozygosity in colon carcinoma from patients with familial polyposis coli. Nature 331: 273–277 (1988).
59. Solomon, E., Voss, R., Hall, V., Bodmer, W. F., Jass, J. R., Jeffreys, A. J., Lucibello, F. C., Patel, I., and Rider, S. H. Chromosome 5 allele loss in human colorectal carcinomas. Nature 328: 616–619 (1987).
60. Dobrovic, A., Houle, B., Belouchi, A., and Bradley, W. E. erbA-related sequence coding for DNA-binding hormone receptor localized to chromosome 3p21-3p25 and deleted in small cell lung carcinoma. Cancer Res. 48: 682–685 (1988).
61. Johnson, B. E., Sakaguchi, A. Y., Gazdar, A. F., Minna, J. D., Burch, D., Marshall, A., and Naylor, S. L. Restriction fragment length polymorphism studies show consistent loss of chromosome 3p alleles in small cell lung cancer patients' tumors. J. Clin. Invest. 82: 502–507 (1988).
62. Kok, K., Osinga, J., Carritt, B., Davis, M. B., van der Hout,

A. H., van der Veen, A. Y., Landsvater, R. M., de Leij, L. F., Berendsen, H. H., Postmus, P. E., Poppema, S., and Buys, C. H. Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. Nature 330: 578–581 (1987).
63. Scrable, H. J., Witte, D. P., Lampkin, B. C., and Cavenee, W. K. Chromosomal localization of the human rhabdomyosarcoma locus by mitotic recombination mapping. Nature 329: 645–647 (1987).
64. Fournier, R. E., and Ruddle, F. H. Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. Proc. Natl. Acad. Sci. U.S.A. 74: 319–323 (1977).
65. Ege, T., Ringertz, N. R., Hamberg, H., and Sidebottom, E. Preparation of microcells. Methods Cell. Biol. 15: 339–357 (1977).
66. Weissman, B. E., Saxon, P.J., Pasquale, S. R., Jones, G. R., Geiser, A. G., and Stanbridge, E. J. Introduction of a normal human chromosome 11 into a Wilms' tumor cell line controls its tumorigenic expression. Science 236: 175–180 (1987).
67. Koi, M., Morita, H., Yamada, H., Satoh, H., Barrett, J. C., and Oshimura, M. Normal human chromosome 11 suppresses tumorigenicity of human cervical tumor cell line SiHa. Mol. Carcinog. 2: 12–21 (1989).
68. Oshimura, M., Koi, M., Morita, H., Yamada, H., Shimizu, M., and Ono, T. Suppression of tumorigenicities of human cancer cell lines following chromosome transfer via microcell fusion. Proc. Am. Assoc. Cancer Res. 30: 786 (1989).
69. Saxon, P. J., Srivatsan, E. S., and Stanbridge, E. J. Introduction of human chromosome 11 via microcell transfer controls tumorigenic expression of HeLa cells. EMBO J. 5: 3461–3466 (1986).
70. Trent, J. M., Stanbridge, E. J., McBride, H. L., Meese, E. U., Casey, G., Araujo, D. E., Witkowski, C. M., and Nagle, R. B. Tumorigenicity in human melanoma cell lines controlled by introduction of human chromosome 6. Science 247: 568–571 (1990).
71. Harris, H., Miller, O. J., Klein, G., Worst, P., and Tachibana, T. Suppression of malignancy by cell fusion. Nature 223: 363–368 (1969).
72. Harris, H. The analysis of malignancy by cell fusion: the position in 1988. Cancer Res. 48: 3302–3306 (1988).
73. Stanbridge, E. J. Genetic regulation of tumorigenic expression in somatic cell hybrids. In: Advances in Viral Oncology, Vol. 6 (G. Klein, Ed.), Raven Press, New York, 1987, pp. 83–101.
74. Peehl, D. M., and Stanbridge, E. J. Characterization of human keratinocyte X HeLa somatic cell hybrids. Int. J. Cancer 27: 625–635 (1981).
75. Geiser, A. G., Der, C. J., Marshall, C. J., and Stanbridge, E. J. Suppression of tumorigenicity with continued expression of the c-Ha-ras oncogene in EJ bladder carcinoma-human fibroblast hybrid cells. Proc. Natl. Acad. Sci. U.S.A. 83: 5209–5213 (1986).
76. Kaighn, M. E., Gabrielson, E. W., Iman, D. S., Pauls, E. A., and Harris, C. C. Suppression of tumorigenicity of a human lung carcinoma line by nontumorigenic bronchial epithelial cells in somatic cell hybrids. Cancer Res. 50: 1890–1896 (1990).
77. Yunis, J. J., and Ramsay, N. Retinoblastoma and subband deletion of chromosome 13. Am. J. Dis. Child. 132: 161–163 (1978).
78. Lee, W. H., Bookstein, R., Hong, F., Young, L. J., Shew, J. Y., and Lee, E. Y. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. Science 235: 1394–1399 (1987).
79. Fung, Y. K., Murphree, A. L., T'Ang, A., Qian, J., Hinrichs, S. H., and Benedict, W. F. Structural evidence for the authenticity of the human retinoblastoma gene. Science 236: 1657–1661 (1987).
80. Knudson, A. G., Jr. A two-mutation model for human cancer. In: Advances in Viral Oncology, Vol. 7 (G. Klein, Ed.), Raven Press, New York, 1987, pp. 1–17.
81. Benedict, W. F. Recessive human cancer susceptibility genes (retinoblastoma and Wilms' loci). In: Advances in Viral Oncology, Vol. 7 (G. Klein, Ed.), Raven Press, New York, 1987, pp. 19–34.
82. Huang, H. J., Yee, J. K., Shew, J. Y., Chen, P. L., Bookstein, R., Friedmann, T., Lee, E. Y., and Lee. W. H. Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. Science 242: 1563–1566 (1988).
83. Mihara, K., Cao, X. R., Yen, A., Chandler, S., Driscoll, B., Murphree, A. L., T'Ang, A., and Fung, Y. K. Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science 246: 1300–1303 (1989).
84. Chen, P. L., Scully, P., Shew, J. Y., Wang, J. Y., and Lee, W. H. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 58: 1193–1198 (1989).
85. Buchkovich, K., Duffy, L. A., and Harlow, E. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell 58: 1097–1105 (1989).
86. DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C. M., and Livingston, D. M. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell 58: 1085–1095 (1989).
87. Ludlow, J. W., DeCaprio, J. A., Huang, C. M., Lee, W. H., Paucha, E., and Livingston, D. M. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. Cell 56: 57–65 (1989).
88. Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature 334: 124–129 (1988).
89. Egan, C., Bayley, S. T., and Branton, P. E. Binding of the Rb1 protein to E1A products is required for adenovirus transformation. Oncogene 4: 383–388 (1989).
90. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E., and Livingston, D. M. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 54: 275–283 (1988).
91. Munger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E., and Howley, P. M. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. 8: 4099–4105 (1989).
92. Dyson, N., Howley, P. M., Munger, K., and Harlow, E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 243: 934–937 (1989).
93. Dunn, J. M., Phillips, R. A., Becker, A. J., and Gallie, B. L. Identification of germline and somatic mutations affecting the retinoblastoma gene. Science 241: 1797–1800 (1988).
94. Dunn, J. M., Phillips, R. A., Zhu, X., Becker, A. J., and Gallie, B. L. Mutations in the RB1 gene and their effects on transcription. Mol. Cell. Biol. 9: 4596–4604 (1989).
95. Yokota, J., Akiyama, T., Fung, Y. K., Benedict, W. F., Namba, Y., Hanaoka, M., Wada, M., Terasaki, T., Shimosato, Y., Sugimura, T., and Terada, M. Altered expression of the retinoblastoma (RB) gene in small-cell carcinoma of the lung. Oncogene 3: 471–475 (1988).
96. Bookstein, R., Shew, J. Y., Chen, P. L., Scully, P., and Lee, W. H. Suppression of tumorigenicity of human prostate carcinoma cells by replacing a mutated *RB* gene. Science 247: 712–715 (1990).
97. Crawford, L. V., Pim, D. C., and Lamb, P. The cellular protein p53 in human tumours. Mol. Biol. Med. 2: 261–272 (1984).
98. Linzer, D. I., and Levine, A. J. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell 17: 43–52 (1979).
99. Mercer, W. E., Nelson, D., DeLeo, A. B., Old, L. J., and Baserga, R. Microinjection of monoclonal antibody to protein p53 inhibits serum-induced DNA synthesis in 3T3 cells. Proc. Natl. Acad. Sci. U.S.A. 79: 6309–6312 (1982).
100. Eliyahu, D., Raz, A., Gruss, P., Givol, D., and Oren, M. Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature 312: 646–649 (1984).
101. Hinds, P., Finlay, C., and Levine, A. J. Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J. Virol. 63: 739–746 (1989).
102. Finlay, C. A., Hinds, P. W., and Levine, A. J. The p53 proto-

oncogene can act as a suppressor of transformation. Cell 57: 1083–1093 (1989).
103. Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kimhi, O,. and Oren, M. Wild-type p53 can inhibit oncogene-mediated focus formation. Proc. Natl. Acad. Sci. U.S.A. 86: 8763–8767 (1989).
104. Lane, D.P., and Crawford, L. V. T antigen is bound to a host protein in SV40-transformed cells. Nature 278: 261–263 (1979).
105. Sarnow, P., Ho, Y. S., Williams, J., and Levine, A. J. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54 kd cellular protein in transformed cells. Cell 28: 387–394 (1982).
106. Burger, C., and Fanning, E. Specific DNA binding activity of T antigen subclasses varies among different SV40-transformed cell lines. Virology 126: 19–31 (1983).
107. Isobe, M., Emanuel, B. S., Givol, D., Oren, M., and Croce, C. M. Localization of gene for human p53 tumour antigen to band 17p13. Nature 320: 84–85 (1986).
108. Sturzbecher, H. W., Addison, C., and Jenkins, J. R. Characterization of mutant p53-hsp72/73 protein-protein complexes by transient expression in monkey COS cells. Mol. Cell. Biol. 8: 3740–3747 (1988).
109. Eliyahu, D., Goldfinger, N., Pinhasi-Kimhi, O., Shaulsky, G., Skurnik, Y., Arai, N., Rotter, V., and Oren, M. Meth A fibrosarcoma cells express two transforming mutant p53 species. Oncogene 3: 313–321 (1988).
110. Lavigueur, A., Maltby, V., Mock, D., Rossant, J., Pawson, T., and Bernstein, A. High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. Mol. Cell. Biol. 9: 3982–3991 (1989).
111. Ehrhart, J. C., Duthu, A., Ullrich, S., Appella, E., and May, P. Specific interaction between a subset of the p53 protein family and heat shock proteins hsp72/hsc73 in a human osteosarcoma cell line. Oncogene 3: 595–603 (1988).
112. Romano, J. W., Ehrhart, J. C., Duthu, A., Kim, C. M., Appella, E., and May, P. Identification and characterization of a p53 gene mutation in a human osteosarcoma cell line. Oncogene 4: 1483–1488 (1990).
113. Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. p53: a frequent target for genetic abnormalities in lung cancer. Science 246: 491–494 (1989).
114. Soussi, T., Caron de Fromentel, C., Mechali, M., May, P., and Kress, M. Cloning and characterization of a cDNA from *Xenopus laevis* coding for a protein homologous to human and murine p53. Oncogene 1: 71–78 (1987).
115. Lehman, T. A., Modali, R., Weston, A., Gerwin, B. I., Ullrich, S., Appella, E., and Harris, C. C. Complex formation of p53 and heat shock proteins in human tumor and transformed cell lines. Am. J. Hum. Genet. (suppl.) 45(4): A204 (1989).